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Localization of Melanoma-Associated Antigen p97 in Cultured Human Melanoma, as Visualized by Light and Electron Microscopy*

WOLFGANG TILGEN, M.D., INGEGERD HELLSTRÖM, M.D., MONIKA ENGSTNER, H. JACQUES GARRIGUES, RÜDIGER RIEHL, PH.D., AND KARL ERIK HELLSTRÖM, M.D.

Universitäts-Hautklinik (WT, ME, RR), Heidelberg, F.R.G., and Fred Hutchinson Cancer Research Center (IH, HJG, KEH), Seattle, Washington, U.S.A.

The expression of a human melanoma-associated antigen, p97, in cultured melanoma cells was investigated using a modification of the Sternberger peroxidase-antiperoxidase (PAP) technique. Explant cultures of two skin melanomas were found to consist of a mixture of cells, some positive and some negative, for the expression of p97. From two other melanomas two cell lines were newly established. All cells from these lines expressed detectable p97 over a period up to 18 months. With the cell lines and the explant cultures we have initiated an investigation of the expression of p97 at the ultrastructural level, using the PAP technique. Antigen expression was detected as a continuous, strongly stained band at the cell surface of the melanoma cells.

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* Dedicated to Prof. Dr. Dr. h.c. U. W. Schnyder on the occasion of his 60th birthday.

Reprint requests to: W. Tilgen, M.D., Universitäts-Hautklinik, Voss-Strasse 2, D-6900 Heidelberg, F.R.G.

Abbreviations:
MEM: minimal essential medium
PAP: peroxidase-antiperoxidase
PBS: phosphate-buffered saline

Monoclonal antibodies to various tumor-associated cell surface antigens of human melanoma have been obtained [1–12]. One of these antigens is p97, a glycoprotein of M_r ~ 97,000 [3,4,13]; the same antigen has been called gp95 by Dippold et al [6]. Studies performed with immunoprecipitation [3,13], absorption [6], and binding techniques [4,13,14] have shown that p97 is strongly expressed (50,000–400,000 molecules/cell) by about 50% of melanomas and more weakly (10,000–50,000 mol-

ecules/cell) by other melanomas. Nonmelanoma tumors contain less than 50,000 molecules of p97 per cell, and normal adult tissues express less than 10,000 molecules per cell [13,14; J. Brown, unpublished findings]. A partial amino acid sequence of p97 has revealed that it is related to transferrin and, like transferrin, p97 can bind iron [15].

In a previous paper [16], Garriques et al reported that p97 can be detected in frozen sections of melanoma biopsies using

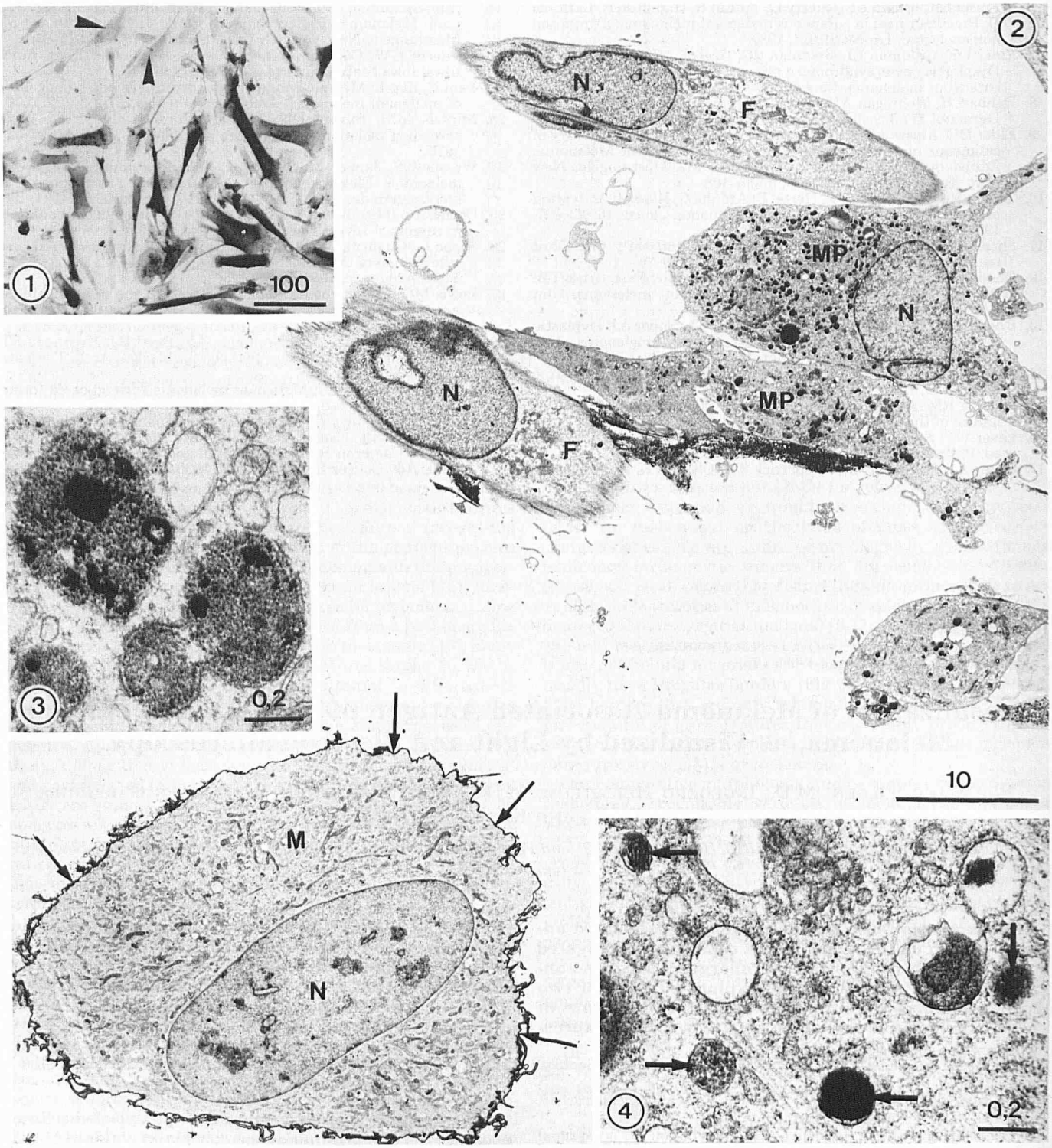


FIG 1. Primary explant culture (27 days) of malignant melanoma after exposure to anti-p97 antibody 96.5. The melanoma cells are darkly stained while fibroblasts (*arrowheads*) show no reaction. *Bar* = 100 μm.

FIG 2. Same explant culture as in Fig 1 exposed to antibody 96.5. The different cell types of the mixed cell population in Fig 1 are clearly distinguishable. *MP* = macrophages, *F* = fibroblasts, both unstained; *M* = melanoma cell outlined by an electron-dense reaction product (*arrows*); *N* = nucleus. *Bar* = 10 μm.

FIG 3. Large lysosomes from macrophages demonstrated in Fig 2. *Bar* = 0.2 μm.

FIG 4. Melanosomes in different degrees of development characteristic for melanoma cells shown in Fig 2. *Bar* = 0.2 μm.

a modification of the Sternberger peroxidase-antiperoxidase (PAP) technique [17]. Binding of anti-p97 antibody was demonstrated with 8 out of 10 primary skin melanomas, with 6 of 7 metastatic melanomas, and with 2 of 2 nevi. No binding was detected with other skin tumors, including basal cell carcinomas, squamous cell carcinomas, and a leiomyosarcoma, nor was binding seen with normal adult skin, except for myoepithelial cells of sweat glands. According to that study [16], and in agreement with binding assays on living cells [3] and immunochemical studies [6,13], p97 is localized at the cell surface.

In this paper, we present more definitive evidence that p97 is strongly expressed at the cell surface, as revealed by an ultrastructural investigation utilizing the PAP technique on cells that were cultured from nodular melanomas of the skin and from nodules arising in superficial spreading melanomas.

MATERIALS AND METHODS

Cell Cultures

Explant cultures were derived from a nodular melanoma and from a nodule within a superficial spreading melanoma. Similarly, 2 cell lines were established from another nodular melanoma and from a nodule within another superficial spreading melanoma. These cell lines were serially transferred in vitro for up to 75 passages during a period of 18 months.

Our cell culture techniques have been described [18] and can be summarized as follows. Tumor biopsy material was dissected as free as possible from connective and necrotic tissue under a stereomicroscope. One-millimeter cubes were placed in plastic Petri dishes and covered with modified Eagle's minimal essential medium (4 × MEM) containing 17% fetal calf serum, after which they were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air; some of the tumor cubes were placed on glass coverslips, but were otherwise treated in the same way. Subcultures were obtained either by trypsinization of attached cells or by aspiration of floating cells. Cultures were regularly tested and found to be free of *Mycoplasma* contamination.

Immunocytochemical Staining

The procedure for the immunocytochemical localization of p97 using the unlabeled antibody technique of Sternberger [17] has been described previously for biopsy material [16]. The cultures used in the present study were processed for immunoreactivity either before or after fixation in the Petri dishes with 1% glutaraldehyde for 30 min and washed with phosphate-buffered saline (PBS) containing 0.01 M lysine. In addition to anti-p97 antibody 96.5 [13] we used antibody H116-22.R as a control. This is a mouse H-2^d anti-H-2^k (IgG 2a) antibody (kindly provided by Prof. G. Hämmerling, German Cancer Research Center, Heidelberg). These antibodies, goat antimouse IgG, and mouse PAP complex were diluted in a PBS solution containing 10% normal human serum and 3% goat serum. Monolayers were incubated with (specific) antibody 96.5 or with (control) antibody H116-22.R, both diluted 1:3 from a pool of hybridoma culture supernatant in a final concentration of 0.166 μg/50 μl, followed by goat antimouse IgG (1:30), and finally by the mouse PAP complex (1:80). Each incubation was for 30 min and after each step, specimens were washed in PBS. The sections were then incubated with freshly prepared 0.05% 3,3′-diaminobenzidine tetrahydrochloride in 0.05 M Tris buffer, pH 7.6, containing 0.01% H_2O_2 for 8 min, in order to develop a visible reaction product, after which the preparations were postfixed with 1% OsO_4 for 20 min.

Immunoelectron Microscopy

Following immunocytochemical staining, cell monolayers in plastic dishes were fixed with 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer containing 50 mM KCl and 2.5 mM $MgCl_2$. Subsequently, the monolayers were postfixed in 2% OsO_4 and stained with 1% aqueous uranyl acetate in situ. Following dehydration in a graded dilution series of ethanol, they were passed through metacrylic acid-2-hydroxypropylester and embedded in Epon 812. After embedding, the cells were removed from the plastic dishes by quick freezing in liquid nitrogen. Ultrathin sections were cut tangentially with a Reichert OmU3 ultramicrotome. Both unstained sections and sections stained with lead citrate were examined with a Philips EM 400 electron microscope.

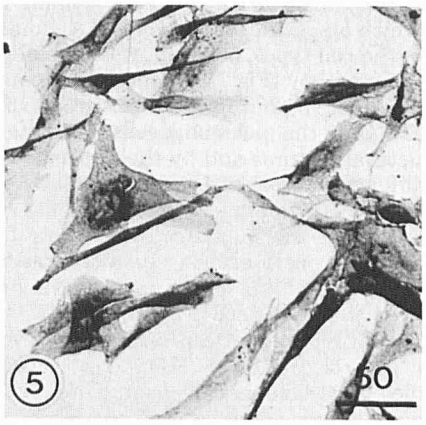
RESULTS

By immunohistochemistry of explant cultures colonies of melanoma cells could be distinguished from contaminating fibroblasts, macrophages, and keratinocytes by staining them with antibody 96.5 which is specific for p97 (Fig 1). An unequivocal identification of the cell types, however, was possible only by immunoelectron microscopy (Fig 2). Macrophages, containing large lysosomes (Figs 2, 3), and fibroblasts were devoid of staining with anti-p97. Only the melanoma cells, characterized by general ultrastructural features and by their specific melanosomes, found in different degrees of development and melanization (Fig 4), showed a dark staining which covered the entire cell membrane (Fig 2). The melanoma cell lines were found immunohistochemically to consist exclusively of cells that, following exposure to antibody 96.5, displayed a dark brown staining which covered the entire cells (Fig 5). This was also true after propagation of one of the cell lines in vitro for 18 months. When the "control" antibody (H116-22.R) was used, only the melanosomes in melanoma cells were darkly stained (Fig 6). Using immunoelectron microscopy, the overall cell architecture, and the cell nuclei, membranes, and organelles, including melanosomes in different degrees of melanization (Fig 7), were well preserved and were comparable in samples exposed to either anti-p97 or control antibody (Figs 8, 9). When the anti-p97 antibody (96.5) was used, an electron-dense reaction product was detected which appeared very closely associated with the cell membrane, where it was heavily and evenly distributed (Fig 8*a*). At higher magnification, this product was found to consist of flocculent, electron-dense aggregates of granular deposits which covered the total cell surface including the interdigitating microvilli (Fig 8*b*). Cultures processed to display immunoreactivity before or after fixation were identical in appearance. No staining was seen in sections exposed to control antibody (Figs 9*a,b*). The cytoplasm and cytoplasmic organelles did not stain with either the anti-p97 or the control antibody.

DISCUSSION

We have investigated the expression of a human melanoma-associated antigen, p97, as defined by a monoclonal mouse antibody, 96.5 [3,13], using a modification of the Sternberger PAP technique [16,17]. The value of the PAP technique for ultrastructural investigations of immunologic phenomena has been well documented in bullous diseases [19,20] and recently described for the identification of Langerhans cells [21] and Sézary cells [22]. Primary cultures and cell lines used in this study were derived from nodular melanomas and nodules arising in superficial spreading melanomas. The explant cultures consisted of a mixed population of cells, including melanoma cells, fibroblasts, macrophages, and keratinocytes, while the cell lines comprised only melanoma cells which exclusively reacted in all cultures with antibody 96.5. In all cases melanoma cells were identified by their contents of melanosomes as specific organelles.

The melanoma cell cultures were chosen for an initial ultrastructural study in which the PAP technique [16,17,23] was used to visualize p97. With this approach, the antigen was detected at the cell surface as a continuous, electron-dense band, while no staining was detected in the cytoplasm. This observation agrees with findings made by light microscopy of sections from melanoma biopsies [16]. In a separate series of experiments the cell lines were also characterized, besides by morphology, by cytometry, protein analysis, and cytogenetics. Tumorigenicity was tested by injecting the cell culture subcutaneously in nude mice. The antigenic phenotype of the melanoma remained stable in the tumors that developed in the sites of injection and in cells recultured from these tumors (Tilgen et al, to be published). The most striking result is that all melanomas so far studied expressed the antigen p97 in the same way, irrespective of the mode and time of propagation and irrespective of the techniques applied. The site of reaction was exclusively restricted to the cell surface. The localization of the human melanoma-associated antigen p97 at the ultrastructural level by the immunoperoxidase method offers a better chance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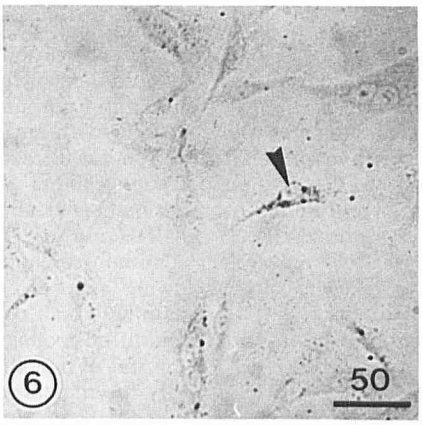
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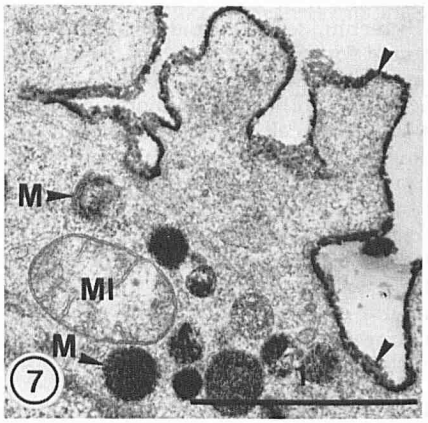
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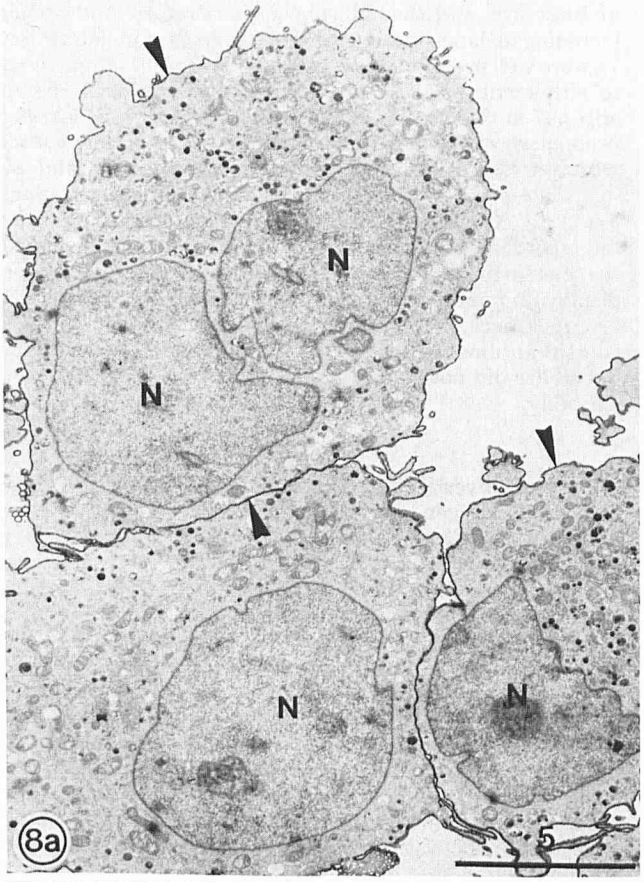
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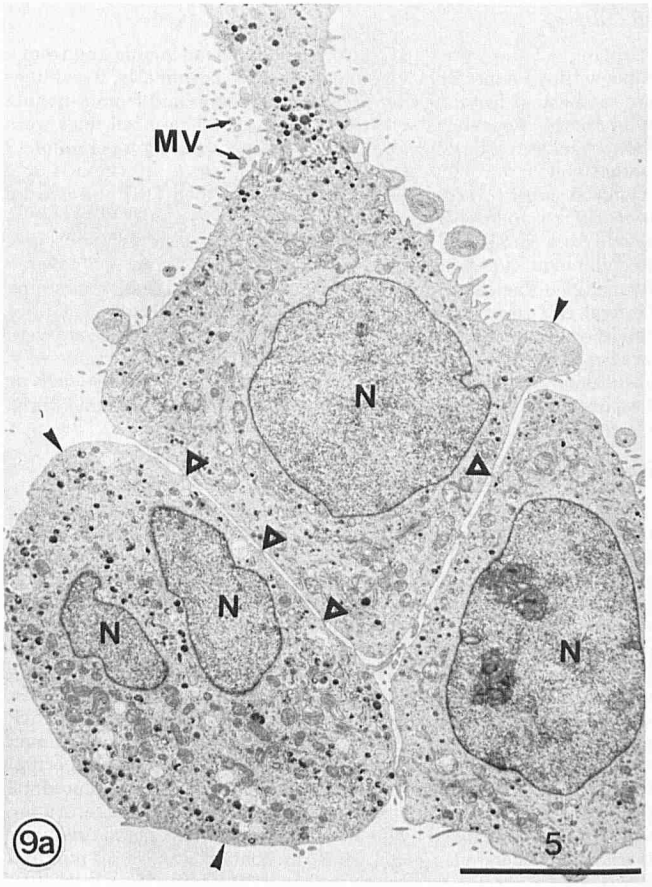
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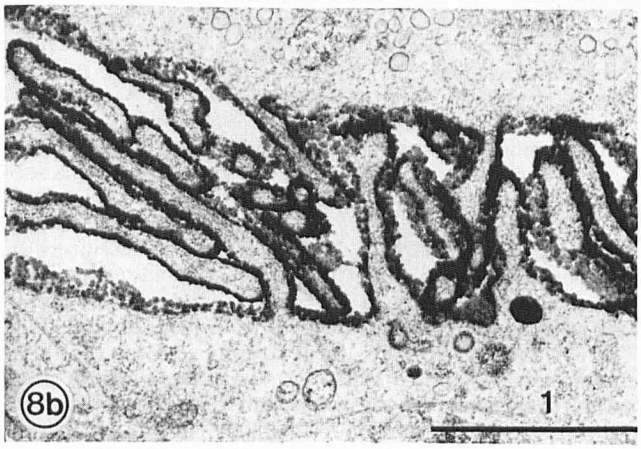
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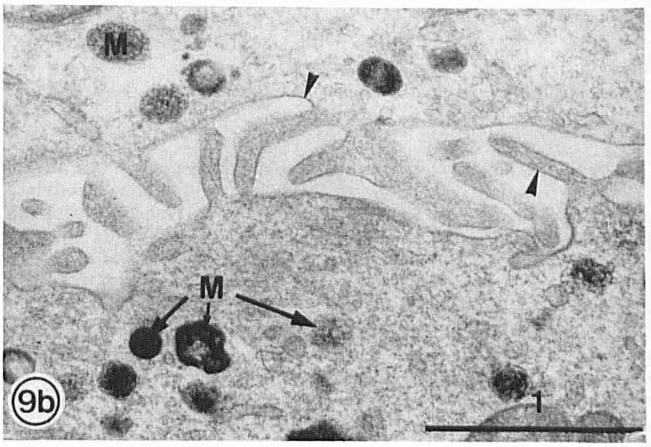
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for determining the exact target and hence the specificity of the monoclonal antibody 96.5.

However, further work is needed in order to detect any expression of p97 in the cytoplasm by exposing fixed and unfixed cells to antibody after they have been sectioned or by treating fixed cells with a lipid solvent to allow penetration of antibody into the cytoplasm. We also plan to use alternative immunochemical procedures which give reaction products with very low solubility, such as labeling antibodies with ferritin. This will allow us to investigate whether p97 is evenly distributed or concentrated in particular areas of the cell membrane.

We feel that our results are relevant for clinical studies utilizing anti-p97 antibodies as carriers of tumor localizing [24] and/or destructive agents by providing direct evidence that p97 is expressed at the cell surface, where it should be easily accessible. In view of the recent evidence that p97 is related to transferrin [15], which is a soluble molecule that is not membrane bound, it is also interesting to note that p97 appears to be a part of the cell membrane.

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FIG 5. Cell line (12 months, 50th passage) of malignant melanoma after exposure to anti-p97 antibody 96.5. All cells reveal a dark brown staining covering the total cell body. *Bar* = 50 μm.

FIG 6. Melanoma cell line as in Fig 5 after exposure to "control" antibody H116-22.R. No staining detectable. The dark melanosomes are indicated by the *arrowhead*. *Bar* = 50 μm.

FIG 7. Melanoma cell line exposed to antibody 96.5. The cell membrane is darkly stained by peroxidase precipitates indicating the site of antigen p97 (*arrowheads*). The cytoplasm contains typical melanosomes (*M*) in various stages of development and melanization. *MI* = mitochondrion. *Bar* = 1 μm.

FIG 8. Melanoma cell line exposed to antibody 96.5. *a*, Melanoma cells are clearly outlined at their surface (*arrowheads*) by an electron-dense reaction product indicating deposition of the antibody. *N* = nuclei. *Bar* = 5 μm. *b*, Flocculent electron-dense aggregates of granular deposits cover the cell membrane including interdigitating microvilli. *Bar* = 1 μm.

FIG 9. Melanoma cell line exposed to "control" antibody H116-22.R. The melanoma cells do not exhibit binding of the control antibody to the cell membrane (*arrowheads*). *N* = nuclei, *MV* = microvilli, *M* = melanosomes in different degrees of melanization. *Open arrowheads* indicate attachment sites of melanoma cells. *a*, *bar* = 5 μm; *b*, *bar* = 1 μm.